Identification of the lichen species in historical orchil dyes by HPLC-MS: a preliminary study

Are traditional neem extract preparations as efficient as the azadirachtin A commercial formulation?

C.M. BOURSIER[a], D. BOSCO[a,b], A. COULIBALY[c], M. NEGRE[a*]

[a]*Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali*
[b]*Centro Interdipartimentale per gli Studi sull'Africa Occidentale (C.I.S.A.O), Università degli Studi di Torino, Via. L. da Vinci 44, 10095, Grugliasco (TO), Italy*
[c]*Institut Polytechnique Rural, Katibougou, Université du Mali*

* Corresponding author. Present address: Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali. Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy.
Tel: 0039 0116708508 Fax: 0033 0116708692.
E-mail address: michele.negre@unito.it

Abstract

Neem tree (*Azadirachta indica* A. Juss) seeds contain many substances with insecticidal properties, the main insecticidal ingredient in the neem-seed extract being azadirachtin A. Several commercial formulations containing azadirachtin A are available on the world market for insect control in organic farming. In developing countries such as Mali, a neem-seed water extract is used to protect organically-grown cotton from both piercing-sucking and chewing insects. The water extract is prepared by soaking ground seeds in water for three or seven days. The scope of this study was to check the effectiveness of the traditional extraction method in terms of azadirachtin A extraction yield and insecticidal activity. The amount of azadirachtin A extracted using the Malian method was 0.19 g/100 g seeds and no significant difference was observed between the rate of extraction after 3 or 7 days. The concentration of azadirachtin A in the seed extract was approximately 200 mg l^{-1}, one order of magnitude higher than the recommended dose of commercial products (25 mg l^{-1}). The extraction rate increased to 0.35 g/100 g seeds when performing three successive water extractions of the ground seeds. A comparison of the extractive capacity of different solvents indicated that the rate of extraction decreased as the polarity of the solvent decreased. The best solvent was water when extraction was performed on whole seeds, while the same

amount of azadirachtin A was extracted by methanol and water from the kernels. Increasing the ionic strength of the water by adding salts did not improve the rate of extraction. The azadirachtin A concentration declined in the extract conserved for more than 3 days at a temperatures higher than 30 °C.

Bioassays were performed on target insects in order to compare the insecticidal activity of the neem extract with that of a commercial product. The bioassays were conducted on the leafhopper *Macrosteles quadripunctulatus,* the noctuid moth *Spodoptera littoralis* and the whitefly *Bemisia tabaci*. The insecticidal preparations were the commercial product Neemazal T/S at the recommended dose of active ingredient (a.i): (25 mg l^{-1}), an aqueous solution of pure azadirachtin at 25 mg l^{-1}, a neem water extract prepared according to the Malian procedure (200 mg l^{-1} a.i) and the same extract diluted to 25 mg l^{-1} a.i. The bioassays conducted on leafhoppers and moths demonstrated that the neem extract at 25 mg l^{-1} a.i. was as effective as the azadirachtin-based commercial product and that the performance of both was higher than that of pure azadirachtin. This result points out the role of the co-formulants of the commercial product and of the co-extracts of the neem-based insecticide. On *B. tabaci*, the efficacy of the water extract at 25 mg l^{-1} a.i. was close to that of pure azadirachtin and lower than that of Neemazal T/S. The same performance as that of Neemazal T/S at 25 mg l^{-1} a.i. was obtained with the water extract at 200 mg l^{-1} a.i.

Keywords: azadirachtin A, neem extract, Neemazal T/S, bioassays

1. Introduction

The increasing interest for bioinsecticides has brought new attention to the neem tree (*Azadirachta indica* A. Juss), already known on the Indian sub-continent for 4000 years (Philogene et al., 2003). The insecticidal activity of some parts of the neem tree is due to limonoids, mainly azadirachtin A and B, nimbin, salannin and similar compounds (Ismam, 2006; Philogene et al., 2003) but most evidence points to azadirachtin as being the most important active principle (Ismam, 2006). Azadirachtin is not persistent in the environment, mainly because of rapid degradation by sunlight. The chemistry, environmental behaviour and biological effects of neem products have been the subject of several reviews (Mordue and Blackwell, 1993; Sundaram, 1996; Veitch et al., 2008).

Several commercial formulations containing azadirachtin A are available on the world market for insect control in organic farming. Azadirachtin-based insecticides are becoming popular in plant protection programmes for cotton because of the worldwide demand for organic cotton (Gahukar, 2000). On the other hand, in developing countries, high cost formulations can not be afforded and neem preparations (neem oil and water extracts) are commonly used as an insecticide.

In Mali, preparations from *A. indica* seeds are used in cotton crops to control insects such as leafhoppers and whiteflies (Hemiptera) which, besides causing direct damage, can also transmit phytoplasma and viruses. The empirical technique used by Malian farmers to produce azadirachtin-based insecticides is based on soaking 100 g seed kernels in 1L water for three to seven days to obtain an aqueous extract.

The insecticidal capacity of commercial formulations of azadirachtin A was assessed through bioassays conducted on target insects such as *Bemisia tabaci* (Gennadius) (Kumar et al., 2005; Kumar and Poelhing, 2006), *Trialeurodes vaporariorum* Westwood (von Elling et al., 2002) and *Spodoptera littoralis* Boisduval (Martinez and van Emden, 2001). Crude water extracts of seeds of the neem tree have been tested in the field against several pests in tropical

and subtropical countries and their efficacy has been found to be satisfactory to excellent (Dreyer and Hellpap, 1991). Several bioassays also attested the activity of natural neem preparations on the noctuid moths *S. littoralis* (Gelbič and Němec, 2001; Sharma et al., 2003), *Spodoptera litura* (F.) (Govindachari et al., 2000; Kumar and Parmar, 1996) and *Peridroma saucia (Hübner),* on the heteropteran bug *Oncopeltus fasciatus* (Dallas) (Isman et al., 1990), the leafhopper *Jacobiasca lybica* (Berg. & Zanon) and the whitefly *B. tabaci* Gennadius (El Shafie and Basedow, 2003).

Although the insecticidal activity of the water extract prepared in Mali has been confirmed in the field and in bioassays (Coulibaly, personal communication), the azadirachtin A concentration of the extract has not been determined and little attention has been given to its storage stability.

The aims of this work were i) to improve the traditional extraction method and conservation of the extracts, ii) to compare the activity of the neem extracts with commercial formulations of neem and of pure azadirachtin A through bioassays on target insects.

2. Materials and methods

2. 1. Neem seeds

Neem seeds were collected in south-western Mali in January 2006, dried at ambient temperature and conserved in the dark, at room temperature. The seeds were ground in a coffee bean blender. When required, the kernels and endocarp were manually separated before grinding.

2.2. Chemicals

All solvents were of analytical or liquid chromatography grade. Standard (99% purity) and technical standard (55%) Azadirachtin A were purchased from Sigma-Aldrich (Milan, Italy). The Neemazal T/S commercial formulation was purchased from Intrachem bio Italia SpA (Grassobbio (BG), Italy).

2. 3. Analysis

2. 3. 1. HPLC analysis

Liquid chromatographic analyses were performed using a Spectrasystem P2000 instrument equipped with a UV detector SpectraSERIES UV100, Thermo Separation Products, St Peters, MO, US) working at 215 nm and a Lichrosphere LC_{18} (25 cm, 4.6 mm, 5 µm) column (Sigma Aldrich, Milan, Italy). The mobile phase was water acidified to pH 3 with H_3PO_4 (67%) and acetonitrile (33%), at 1 ml min^{-1}. The retention time of azadirachtin A was 9.5 min.

2. 3. 2. LC-MS/MS analysis

The LC peak was confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The analyses were performed using a Varian 310 triple quadrupole mass spectrometer (Varian, Italy) equipped with an electrospray ionization ESI source, a 212 LC pump and dedicated software. Separation was performed on a Pursuit 5 C_{18} column (3 µm, 150 mm × 2.0 mm) (Varian, Italy). The mobile phase consisted of 20% water and 80% acetonitrile, both containing 0.3% (V/V) acetic acid delivered at a flow rate of 0.2 ml min^{-1}.

Mass spectrometric analyses were performed in the negative-ion mode, the nebulising gas was N_2 (25 psi, the drying gas was air (250 °C, 25 psi), the capillary voltage was – 35 kV and the collision gas was argon set at 1.8 mTorr. The respective ion transitions were as follows: m/z 719 → 485 (collision energy 14 V), m/z 719 → 535 (collision energy 16 V).

2.4. Preparation of neem extracts

2. 4. 1. Extraction with different solvents

Extraction with water, organic solvents (methanol, acetonitrile and ethyl-acetate) and aqueous salt solutions (0.1 M potassium chloride, calcium nitrate, and calcium chloride) were performed using the following procedure: samples of seeds (1 g) were blended with pure solvent or solution (10 ml) in a mechanical stirrer for 30 min and the supernatant was separated by centrifugation (15 min, 3000 rpm). The extraction was repeated twice and the supernatants were pooled in a 50 ml flask, and then diluted with acetonitrile (1:1, V/V). The same procedure was used for the water extraction of the kernels and woody endocarp. All of the experiments were run in triplicate.

2. 4. 2. Extraction kinetics

Samples of ground seeds (1 g) were blended with water (10 ml) in the dark for 1 h, 24 h, 3 d and 7 d. A single extraction was made, as in Mali. At each sampling point, the supernatant was separated and analysed as indicated above. All of the experiments were run in triplicate.

2. 4. 3. Effect of temperature and storage time on the stability of the extract obtained using the Malian method

Samples of ground seeds (10 g) were extracted with water (100 ml) for 3 days in the dark at room temperature. The supernatants were separated by centrifugation then stored at 14, 25, 30, and 40 °C. After 0, 1, 24, 76 and 168 h, an aliquot of each sample was analysed to determine the azadirachtin A concentration. All of the experiments were run in triplicate

2. 5. Bioassays

2. 5. 1. Insecticidal preparation**.**

The recommended dose of Neemazal/TS is 2-3 L / ha at the dilution rate of 200-300 ml/ hl. Since the product contents 10 g/L azadirachtin A, 200-300 ml/ hl correspond to 20-30 mg l^{-1} a.i. An intermediate concentration of 25 mg L^{-1} azadirachtin A has been used to prepare the insecticidal preparations tested on the insects (pure azadirachtin A, Neemazal T/S, and a neem water extract). A seed water extract was also prepared with 200 mg l^{-1} azadirachtin A. Standard azadirachtin A was used to prepare a solution at a concentration of 25 mg l^{-1}. A volume of 2.5 ml Neemazal/TS was dissolved in 1l water in order to obtain an active ingredient (a.i.) concentration of 25 mg l^{-1}. Ground seeds (10 g) were blended with water (100 ml) for 3 days and the mixture filtered on a Whatman N°4 filter. The filtrate was diluted with acetonitrile (1:1, V/V) then analysed by HPLC. When required, the concentration was adjusted by water dilution.

2. 5. 2. *Macrosteles quadripunctulatus.*

The treatment was performed by spraying oat plants with the insecticide solutions (3-6 pots per trial). Ten 3^{rd} - 4^{th} instar nymphs were caged inside glass cylinders on the potted plants. A control trial was conducted under the same conditions but this time feeding leafhoppers on water-treated plants. After 25 days the adults which emerged from nymphs were counted.

2. 5. 3. *Bemisia tabaci*.

Twenty females were caged on one cucumber plant at the 4-6 leaf stage for an oviposition period of 3 days (three plants per trial). The females were removed and 10 days after the beginning of the oviposition period the treatment was applied. As soon as the adults emerged they were counted and removed. A control trial was carried out by spraying the plant with

water; the activity of the different treatments was expressed as the number of emerged adults and as the mortality rate vs. control treatment.

2. 5. 4. *Spodoptera littoralis.*

The treatment was performed by spraying the insecticide solutions on broad bean plants (three plants per trial) placed in Plexiglas isolators. Ten 3^{rd}- 4^{th} instar larvae were placed on each plant. The diet was completed by fresh, untreated sting bean when all the treated plants were consumed. A control trial was conducted under the same conditions but feeding larvae with water-treated plants. The insecticidal activity was evaluated as larval mortality.

All of the bioassays were performed in climatic chambers with temperatures ranging from 20 to 25 °C and a 16:8 L:D photoperiod.

Bioassay data were evaluated by one-way ANOVA ($P<0.001$) followed by the Holm-Sidak method for Multiple Comparison Procedures.

3. Results and discussion

3. 1. Yield from the extraction of azadirachtin A from neem seeds and kernels

Azadirachtin A is known to be soluble in polar organic solvents and ~~slightly~~ ~~soluble~~ in water (1.29 g l^{-1}) (Mordue et al., 2004; Tomlin, 2006). Traditional African and Indian neem-based insecticides are prepared by water extraction of neem seeds or neem kernels, and the recovery of azadirachtin by such methods has not been fully investigated. The extraction of the neem triterpenoids reported in literature includes either first grinding the seeds with an alcohol and then removing the oil by partitioning the alcoholic extract against a more lipophilic solvent, or alternatively grinding the seeds first with hexane or petrol to remove the oil, and then extracting the triterpenoids from the seed matrix using a more polar solvent (Jarvis et al., 1999). Aqueous solutions are usually made by first dissolving azadirachtin obtained through

the processes described above in ethanol or acetone and then carefully diluting with water (Mordue et al., 2004).

In order to compare the efficacy of the water extraction with that of organic solvents, three successive extractions of powered neem seeds were performed with different solvents and salt solutions. The amount of extracted azadirachtin A expressed in g/100 g dried seeds was as follows: water 0.35 (SE = 0.06), methanol 0.24 (SE = 0.02), acetonitrile 0.11 (SE = 0.02) and ethyl acetate 0.05 (SE = 0.01), therefore the amounts of extracted azadirachtin decreased with decreasing polarity of the solvent. The extractive capacities of the salt solutions (potassium chloride, calcium nitrate, and calcium chloride) were not significantly different from that of water, indicating that the ionic strength of the solution did not affect the recovery.

The amount of azadirachtin A recovered from the seeds by water and methanol extraction was in the range reported by Jarvis et al. (1999): 0.2-0.8 g/100 g seeds, ~~taking into account that the amount of azadirachtin~~ depending on the provenance of the seeds (Ismam et al., 1990). On the other hand, the fact that extraction with water lead to a recovery higher than with methanol was in contrast to expectations since azadirachtin A is more soluble in methanol than in water but, to the best of our knowledge, no data concerning the yield from water extraction of azadirachtin from whole neem seeds is reported in the literature.

The same extraction procedure (limited to water and methanol) was also applied to the two main parts of the seeds, the kernel and the woody endocarp. The results illustrated in Table 1 show that the fraction of azadirachtin A contained in the endocarp was negligible (<0.02 g/100 g), confirming that the active ingredient was concentrated in the kernel. The yield from the kernel extraction with methanol (0.40 g/100 g kernels) agrees with the data in the literature: Mordue et al. (2004) reported that the amount of azadirachtin A extractable from seed kernels with solvent ranges between 0.1 and 1 g/100 g (mean 0.6 g/100 g).

Kleeberg and Ruch (2006) analysed hundreds of samples where the content of azadirachtin A ranged from 3 to 9.6 g /Kg kernels.

Extraction of the kernels with water lead to a recovery which was not significantly different to that with methanol. The surprisingly high recoveries obtained by water extraction of the kernels cannot be compared with data in the literature because it has seldom been investigated. Govindachari et al. (1999) found 2.15 mg azadirachtin A/100g kernels in an aqueous extract. Such a low recovery could be attributed to the loss of the active ingredient due to the complex extraction procedure used by these authors. Since the kernel accounts for approximately 50% (w/w) of the seed and the amount of azadirachtin A in the endocarp is negligible, the expected amount extracted from 100 g kernels should be about twice the amount extracted from 100 g seeds. This occurred for the methanol extraction but not for the water extraction, which gave a proportionally lower recovery from the kernels than from the seeds. One reason for this could be that the presence of the kernels allows a better grinding of the seed, therefore the exposed surface area of the seeds is higher than that of the kernels, which promotes the extractive capacity of the water. Another possible reason is that the kernel contains a higher concentration of oil, making the kernel powder more hydrophobic than ground whole seeds.

3.2. Extraction kinetics

Since the traditional Malian extraction procedure includes relatively long extraction times (3 or 7 days), the amount of azadirachtin A recovered from neem seeds by a single water extraction was measured at different time intervals. The quantity recovered after 1 h was 0.19 g/100 g (SE = 0.02) and did not improve significantly with longer extraction times. Considering that the quantity obtained with three successive extractions (see above) was 0.34 g/100 g (SE = 0.06), we can conclude that the single extraction performed in the Malian

method is not exhaustive. On the other hand, the concentration of azadirachtin A in the aqueous phase was 200 mg l^{-1}, therefore much higher than the recommended rate (25 mg l^{-1}).

3. 3. Stability of the extract

The effect of storage times and temperatures on the concentration of azadirachtin A in neem preparation obtained by extraction of seeds for 3 days was tested in order to check the stability of the solution stored in the dark. The results, reported in table 2, attest to the stability of the extract for at least 7 days at 14 and 25 °C. At 30 °C the azadirachtin A concentration decreased by about 30% of the initial concentration between the third and the seventh day. At 40 °C, more than 20% of the azadirachtin A degraded after 1 h and only 30% was still present after 7 days. Since the experiment was conducted in the dark, the main factors controlling the deasapparance of azadirachtin A in the aqueous solution, besides thermodegradation, would have been chemical and biological factors. Microbial degradation would have plaid a minor role as attested by a study of kinetics conducted in sterile and unsterilized natural waters (Sundaram, 1996). Chemical degradation of azadirachtin is promoted by basic pH (Barrek et al., 2004; Sundaram, 1996; Szeto and Wan, 1996). The latter mentioned authors studied the effect of temperature (between 40 and 70 °C) at different pH values on the degradation of azadiractin A in the dark. They observed that the rate of disappearance of azadirachtin A increased with temperature independently of pH. At pH 4, which is the pH of the neem aqueous extract, Barrek et al. (2004) reported an half-life of azadirachtin of 16 days at 40 °C, while the degradation rate at the same temperature in the neem extract was more rapid (table 2). This suggests that the presence of the other components of the extracts contributes to the minor stability of azadirachtin A.

Since countries like Mali normally experience high temperatures (up to 40 °C in the hot season in the south and up to 50 °C in the north), conservation of the water-extracted

insecticidal solutions, although in the dark, is not advisable unless a low temperature can be guaranteed. Another risk factor for the loss of the active ingredient is exposure to sunlight, which has been seen to dramatically increase the degradation rate to half-time values of a few hours (Caboni et al., 2009).

3. 4. Bioassays

The insecticide activity of the commercial formulation Neemazal T/S, of pure azadirachtin A and of traditional water extracts against *M. quadripunctulatus*, *B. tabaci* and *S. littoralis* are summarized in Tables 3, 4 and 5.

The efficacy of the insecticide treatments against *Macrosteles quadripunctulatus* is expressed as mortality rate. The activity of the water extract containing 25 mg l^{-1} azadirachtin A was the same as the commercial product (83-86% mortality) at the same concentration. The mortality rate of the extract at 200 mg l^{-1} a.i. reached 97%, confirming that the traditional Malian preparation is highly active against this pest. In contrast, the mortality rate of the trial conducted with pure azadirachtin A was very low, at 30%. The good performance of the commercial formulation and of the neem extract is in agreement with some data reported on other leafhoppers: Meisner et al. (1992) observed that Margosan-o, a neem-based insecticide was active at a concentration of 30 mg l^{-1} against *Asymmetrasca decedens* , limiting the growth of 90% of the nymphs. Soil application of neem cake and foliar spray of a neem kernel water extract in a rice field resulted in a decrease of about 46% of the population of *Nephotettix virescens* at 5 days after treatment (Rajappan et al., 2000).

The activity of the neem water extract and of the pure active ingredient at 25 mg l^{-1} against *B. tabaci* was lower compared to the commercial product. The good performance of Neemazal TS is in agreement with the results of Kumar et al. (2005), who found 89% mortality of *B. tabaci* 1^{st} instar nymphs following foliar treatment with the same product, at a

very similar concentration. El Shafie and Basedow (2003) compared the insecticidal activity against *B. tabaci* Gennadius of a commercial formulation, a neem oil and a neem kernel water extract (50 g ground fresh neem seeds per litre). They observed that neem oil was effective and assumed that oil promotes the effect of azadirachtin A, for example by improving uptake through the cuticle. As far as the water extract was concerned, although the azadirachtin concentration was not measured, the authors concluded that more neem seeds would be needed to produce effects similar to those of the commercial product. This is in agreement with our assays, which indicated that *B. tabaci* is very sensitive to a high concentration of neem water extract, 200 mg l^{-1}, and in fact, farmers in Mali apply such high water extract concentrations against this pest.

The number of dead *S. littoralis* larvae was determined 14 days after treatment. After this date, no changes were observed. The neem extract trial showed 100% mortality, while the performance of pure azadirachtin was lower, at 83%. In some cases, the larvae grown on the treated plants were smaller and had a different pigmentation than those grown on untreated plants. Azadirachtin is known to cause malformation on *S. littoralis* larvae at different development stages (Martinez and van Emden, 2001; Gelbič and Němec, 2001; Nathan and Kalaivani, 2006).

For both *M. quadripunctulatus* and *S. littoralis*, the water extract and commercial formulation were more effective than the pure azadirachtin. This effect was more evident in the *M. quadripunctulatus* bioassay where the pure product did not exhibit any insecticidal activity.

The fact that pure azadirachtin was less effective than the neem extract and the commercial formulation against leahoppers and moths suggest that other components present in these preparations contribute to the insecticidal activity. The performance of the commercial formulation is improved by the addition of co-formulants and co-adjuvants. These

compounds indirectly contribute to the insecticide activity by protecting the active ingredient against degradation (mainly photolytic) and by improving contact with the treated leaves. As far as the neem extract is concerned, although we only measured azadirachtin A, a large amount of data in the literature attests the presence of other terpenoids which could act as insecticides and/or improve the effectiveness of azadirachtin A through a synergistic effect of co-extracted compounds.

4. Conclusions

The Malian empirical water extraction technique from neem seeds provides insecticidal preparations containing about 200 mg l^{-1} azadirachtin A, which is much higher than the recommended application rate of commercial formulations. Consequently, the improvement in the extraction yields which can be obtained by several successive extractions of the same seeds does not appear to be useful for field applications considering that neem trees are largely available in Africa. On the other hand, insecticide treatments should be performed with freshly-prepared neem extracts or extracts conserved at low temperatures to avoid degradation of the active ingredient.

The bioassays conducted on leafhoppers and moths showed that the neem extract was as effective as the azadirachtin-based commercial product and that the performance of both insecticide preparations was higher than that of pure azadirachtin. In the case of the commercial product, such a result can be attributed to the presence of co-formulates which improve the stability of the active ingredient and its contact with treated leaves. In the case of the neem extract, its efficacy is most likely due to a matrix effect or to the presence of other terpenoids.

In contrast, the efficacy of the neem extract on *B. tabaci* was significantly lower than that of the commercial product at the recommended dose. A more concentrated extract was

needed to obtain the same performance as the commercial product. The minor performance of the neem extract at the lowest tested concentration could be due to the fast degradation rate of the active ingredient, limiting its contact time with the insects.

5. Acknowledgments

This work was supported by the grant “Cooperazione interuniversitaria Torino-Sahel” in the Piemonte Region.

6. References

Barrek, S., Paisse, O., Grenier-Loustalot, M.F., 2004. Analysis of neem oils by LC–MS and degradation kinetics of azadirachtin-A in a controlled environment. Characterization of degradation products by HPLC–MS–MS. Anal. Bioanal. Chem. 378, 753–763.

Caboni, P.L., Sarais, G., Angioni, A., Lai, F., Dedola, F. Cabras, P., 2009. Fate of azadirachtin A and related azadirachtoids on tomatoes after greenhouse treatment. J. Environ. Science Health, Part B. 44, 598–605.

Dreyer, M., Hellpap, C., 1991. Niem – ein vielversprechendes natürliches insektizid für kein-flächige gemüseproduktion in tropischen und subtropischen ländern. Seitschrift für Pflanzenkrankheiten und Pflanzenschutz. 98(4), 428–437.

El Shafie, H.A.F., Basedow, T., 2003. The efficacy of different neem preparation for control of insects damaging potatoes and eggplants in the Sudan. Crop Protection. 22, 1015–1021.

Gahukar, R.T., 2000. Use of neem products/pesticides in cotton pest management. Int. J. Pest manage. 46, 149–160.

Gelbič, I., Němec, V., 2001. Developmental changes caused by metyrapone and azadirachtin in *Spodoptera littoralis* (Boisd.) (Lep., Noctuidae) and *Galleria melonella* (L.) (Lep., Pyralidae). J. Appl. Ent. 125, 417–422.

Govindachari, T.R., Gopalakrishnan, G., Suresh, G., 1999. Triterpenoidal constituent of an aqueous extract from neem kernel. Fitoterapia. 70, 558–560.

Govindachari, T.R., Suresh, G., Gopalakrishnan, G., Wesley, S.D., 2000. Insect antifeedant and growth regulating activities of neem seed oil- the role of major tetrenotriterpenoids. J. Appl. Ent. 124, 287–291.

Isman, M.B., Koul, O., Luczynski, A., Kaminski, J., 1990. Insecticidal and antifeedant bioactivities of neem oils and their relationship to azadirachtin content. J. Agric. Food Chem. 38, 1408–1411.

Isman M.B., 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu. Rev. Entomol. 51, 45–66.

Jarvis, A.P., Morgan, E.D., Edwards, C., 1999. Rapid separation of triterpenoids from neem seeds extracts. Phytochem. Anal. 10, 39–43.

Johnson, S., Morgan, D., Peiris, C.N., 1996. Development of the major triterpenoids and oil in the fruit and seeds of neem (*Azadirachta indica*). Ann. Bot. London. 78, 383–388.

Kleeberg, H., Ruch, B., 2006. Standardization of Neem-extracts. Proceedings of Inc2006, Kunming, China. Nov. 2006

Kumar, P., Parmar, B.S., 1996. Physicochemical and chemical variation in neem oils and some bioactivity leads against *Spodoptera litura* F. J. Agric. Food Chem. 44, 2137–2143.

Kumar, P., Poehling, H.M., Borgemaister, C., 2005. Effect of different application method of azadirachtin against sweet potato whitefly *Bemisia tabaci* Gennadius (Hom., Aleyrodidae) on tomato plants. J. Appl. Entomol. 129,489–497.

Kumar, P., Poehling, H.M., 2006. Persistence of soil and foliar azadirachtin treatments to control sweet potato whitefly *Bemisia tabaci* Gennadius (Hom., Aleyrodidae) on tomato under controlled (laboratory) and field (netted greenhouse) conditions in humid tropics. J. Pest. Sci. 79, 189–199.

Martinez, S.S., van Emden, H.F., 2001. Growth disruption, abnormalities and mortality of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) caused by azadirachtin. Neotrop. Entomol. 30, 113–125.

Meisner, J., Klein, M., Ben-Moshe, E., 1992. Effect of Margosan-O on the development of the leafhopper *Asymmetrasca decedens*. Phytoparasitica. 20, 15–23.

Mordue, A.J., Blackwell, A., 1993. Azadirachtin – an update. J. Insect Physiol. 39, 903–924.

Mordue, A.J., Morgan, E.D., Nisbet, A.J., 2004. Azadirachtin, a natural compound in insect control, in: Kostas, I., Gilbert, L., Sarjeet, G. (Eds), Comprehensive Molecular Insect Science. Elsevier Pergamon, Oxford, pp. 117–135.

Nathan, S.S., Kalaivani, K., 2006. Combined effects of azadirachtin and nucleopolyhedrovirus (SpltNPV) on *Spodoptera littoralis* Fabricius (Lepidoptera: Noctuide) larvae. Biol. Control. 39, 96–104.

Philogène, B.J.R., Regnault-Roger, C., Vincent, C., 2003. Produits phytosanitaires insecticides d'origine végétale : promesses d'hier et d'aujourd'hui, in: Regnault-Roger, C., Philogène, B.J.R.,Vincent, C. (Eds), Biopesticides d'origine végétale. TEC & DOC, Paris, pp. 1–15.

Rajappan, K., Ushamalini, C., Subramanian, N., Narasimhan, V., Abdul Kareem, A., 2000. Effect of botanicals on the population dynamics of *Nephotettix virescens*, rice tungro disease. Incidence and yield of rice. Phytoparasitica. 28, 109–113.

Sharma, P.R., Sharma, O.P., Saxena, B.P., 2003. Effect of Neem gold on haemocytes of tobacco armyworm, *Spodoptera littoralis* (Fabricius) (Lepidoptera; Noctuidae) Curr. Science India. 84, 690–695.

Sundaram, K.M.S., 1996. Azadirachtin biopesticide: A review of studies conducted on its analytical chemistry, environmental behaviour and biological effects. J. Environ. Sci. Health, Part B, 31, 913–948.

Szeto, S.Y., Wan, M.T., 1996. Hydrolysis of azadirachtin in buffer and natural water. J. Agric. Food Chem. 44, 1160–1163.

Tomlin, C.D.S., 2006. The Pesticide Manual, Fourteenth Edition, BCPC Publisher, Alton, pp. 46–47.

Veitch, G.E., Boyer, A., Ley, S.V., 2008. The azadirachtin story. Angew. Chem., Int. Edit. 47, 9402–9429.

von Elling, K., Borgemaister, C., Sètamou, M., Poehling, H.M., 2002. The effect of NeemAzal-T/S®, a commercial product, on different developmental stages of the common greenhouse whitefly *Trialeurodes vaporariorum* Westwood (Hom. Aleyrodidae). J. Appl. Entomol. 126, 40–45.

Figure 1. Effect of temperature on the disappearance of azadirachtin A in the neem aqueous extract.

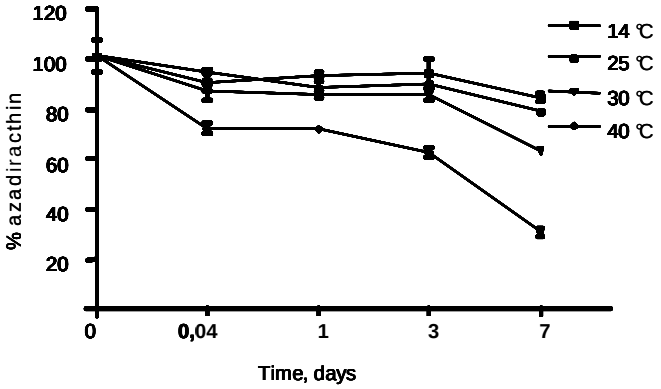

Table 1. Recovery of azadirachtin A from neem seeds, kernel and woody endocarp by water and methanol extraction.

	Water mg/100 g (SE)	Methanol mg/100 g (SE)	
Seed	0.34 (0.06)	0.24 (0.02)	P = 0.205
Kernel	0.42 (0.06)	0.40 (0.01)	P = 0.756
Woody endocarp	< 0.02	0.02 (0.00)	

Table 2. Activity of different azadirachtin A preparations against the leafhopper *Macrosteles quadripunctulatus*. Within column, values followed by the same letter are not significantly different (P<0.001).

Treatments	Mortality rate, % (SE)
Water treated	11.4 (5.1) a
Neemazal T/S 25 mg L^{-1}	85.7 (2.0) b
Pure azadirachtin A 25 mg L^{-1}	30.0 (5.8) c
Neem extract 25 mg L^{-1}	83.3 (3.3) b
Neem extract 200 mg L^{-1}	97.5 (2.5) e

Table 3. Activity of different azadirachtin A preparations against the whitefly *Bemisia tabaci*.

Treatments	*N° of emerged adults*	*Mortality rate vs control, %*
Water treated	808	-
Neemazal T/S 25 mg L^{-1}	48	94.1
Pure azadirachtin A 25 mg L^{-1}	139	82.8
Neem extract 25 mg L^{-1}	209	74.1
Neem extract 200 mg L^{-1}	0	100

Table 4. Activity of different azadirachtin A preparations against the moth *Spodoptera littoralis* 14 days after treatment. Within column, values followed by the same letter are not significantly different ($P<0.001$).

Treatments	Mortality rate, % (SE)
Water treated	33.3 (5.8) a
Neemazal T/S 25 mg L^{-1}	100.0 b
Neem extract 25 mg L^{-1}	100.0 b
Pure azadirachtin A	83.3 (5.8) c